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Award Number: W81XWH-07-1-0303

TITLE: COX-1 Suppression and Follicle Depletion in the Etiology of Menopause-Associated Ovarian Cancer

PRINCIPAL INVESTIGATOR: Martin G. Belinsky, Ph.D.

CONTRACTING ORGANIZATION:
Fox Chase Cancer Center
Philadelphia, PA 19111

REPORT DATE: October 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3 DATES COVERED *(From - To)*
01-10-2009	Final	01 Apr 2007 - 30 Sep 2009

4. TITLE AND SUBTITLE

COX-1 Suppression and Follicle Depletion in the Etiology of Menopause-Associated Ovarian Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-07-1-0303

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Martin G. Belinsky, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fox Chase Cancer Center

Philadelphia, Pennsylvania 19111
E-Mail: Martin.Belinsky@fccc.edu

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

✓ Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Menopause is defined as a permanent cessation of menstruation resulting from depletion of germs cells and loss of ovarian follicular activity. Menopausal ovaries undergo morphological changes that are likely related to the increased risk of ovarian cancer in the peri- and post-menopausal periods. The germ cell-deficient Wv mice recapitulate these post-menopausal alterations in ovarian morphology and develop tubular adenomas. Genetic deletion of cyclooxygenase 1 (Cox-1) in the Wv/Wv background reduced the tumor lesions nearly 3-fold in 4 month mice. Moreover, Cox-1 deletion appeared to delay maturation of small preantral follicles, thus delay follicle depletion and subsequently delay the tumor development. Pharmacological inhibitors of Cox-1 also rescued the tumor phenotype and preserved primary follicles in aged mice. These findings suggest that Cox-1 activity may contribute to preneoplastic morphological changes of the ovarian surface epithelium, which can potentially be prevented by pharmacological inhibitors of Cox-1. Moreover, the observations indicate that depletion of follicles may underlie the etiological factors that influence ovarian cancer risk.

15. SUBJECT TERMS

Ovary, cyclooxygenase-1, tumor, follicle

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Martin Belinksy, Ph.D.
Unclassified	Unlimited	Unclassified		13	19b. TELEPHONE NUMBER: 215-728-2756

Table of Contents

Final Report: **Ovarian Concept Award W81XWH0710303**

Title: **COX-1 suppression and follicle depletion in the etiology of menopause-associated ovarian cancer**

PI: **Martin Belinsky, Ph.D.**

Introduction

The research funded by this Ovarian Concept Award explored the hypothesis that the gonadotropin-stimulated inflammation-like reaction that occurs during the peri- and immediate post-menopause periods causes remodeling and morphological changes that promote ovarian cancer by selecting tumor-prone cells. Cox-1 is most likely involved in the degeneration of ovarian follicles, and inhibition of Cox-1 may reduce the gonadotropin levels and preserve ovarian function. Preservation of follicles may delay menopause and reduce the risk of ovarian cancer. Three specific aims were proposed: ***Aim 1***) Genetic analysis to determine if Cox-1 deletion alters tumor formation and the rate of follicle degeneration; ***Aim 2***) Targeted Cox inhibition to determine whether Cox-1 is selective for follicle atresia and tumor phenotype; and ***Aim 3***) Evaluation of Cox-1 depletion/inhibition on ovarian physiology.

The experiments have established and studied a mouse model that incorporates postmenopausal biology and ovarian function in establishing ovarian cancer etiology and risk. Three mouse lines were established and/or maintained for these experiments: 1) Wv mutant mice (C57BL/6J-Kit$^{W\text{-}v/J}$ from Jackson Laboratory) contain a point mutation in the kinase domain of c-Kit, are viable, and lack pigment-forming cells, germ cells, red blood cells, and mast cells. The females are sterile, and their ovaries have an aged, menopause-like phenotype. 2) Cox-1 homozygous mutant mice, from Langenbach and colleagues (NIEHS, Research Triangle Park, NC), develop normally, have no gastric pathology, are fertile, but have been reported to have parturition defects. 3) Wv were intercrossed with Cox-1 mutant mice to generate Wv/Wv:Cox-1(-/-).

Body

Although the etiology of ovarian cancer is complex and not completely understood, several significant factors have been identified that influence the ovarian cancer risk. In particular, age—even more so than family history for ovarian cancer—is the strongest predictor of risk (1). Approximately 85% of ovarian cancer is diagnosed in post-menopausal women, and the average age of diagnosis (~54 years) is in the immediate post-menopausal period (~52 years). This suggests a strong correlation between menopausal status and ovarian cancer risk.

Menopause is defined as the cessation of ovulation and menstruation and is caused by the loss of oocytes and follicular function (2). Most oocytes are progressively lost by atresia, or apoptosis (3), and the ovary may develop a number of atrophic features due to its aging and loss of function as an endocrine gland (4). One such consequence is the unopposed increase in gonadotropin levels. In the pre-menopausal ovary, gonadotropins stimulate expression of cyclooxygenases, bifunctional enzymes that convert arachidonic acid to prostaglandin H2, which is subsequently converted to other prostaglandins by specific prostaglandin synthases. The prostaglandins stimulate an inflammatory-like condition that results in proteolysis responsible for egg release from the ovary (5). Progesterone produced by ovarian corpus luteum feeds back to inhibit gonadotropin release by the pituitary. In the post-menopausal ovary, corpora lutea do not form (because of a lack of follicle predecessors and ovulation) and progesterone is not produced. As a result, gonadotropins are highest in the post-menopausal period. These hormones may still stimulate ovulation-like processes such as proteolytic

degradation and influence cyclooxygenase enzyme expression in the ovarian surface epithelial and follicular granulosa cells. This inflammation-like condition stimulated by the elevated level of gonadotropins is likely a primary reason for the increased risk of ovarian cancer (6).

We examined the naturally occurring mutant mouse, the white spotting variant (Wv) mice to understand the relationship between the development of age-related morphological changes in the ovary and depletion of ovarian oocytes and follicles. These mice harbor a point mutation in the kinase domain of the c-kit gene, resulting in developmental defects in germ cells, pigment-forming cells, red blood cells, and mast cells in homozygous mutant mice (7-9). At birth Wv homozygous mutant ovaries contain less that 1% of the normal number of oocytes, and the remaining oocytes are depleted by about 6-8 weeks of age (10). Consequently, ovulation ceases and levels of serum pituitary gonadotropins increase. Pronounced epithelial morphological changes develop, including surface invaginations, inclusion cysts, papillomatosis, and benign ovarian tumors, known as tubular adenomas that completely infiltrate the ovary by four months of age (9,11). The tumors are derived from ovarian surface epithelial cells, resembling human ovarian changes that may result from aging (11). Gonadotropins are believed to be the cause of these tubular adenomas, since suppression of gonadotropin release in Wv mice prevents the development of the ovarian tubular adenomas (12). The Wv mice over-express cyclooxygenase 1 (Cox-1) and cyclooxygenase 2 (Cox-2) (11), which are distinct enzymes expressed either constitutively or induced, respectively. The COX enzymes regulate multiple aspects of female reproduction (13), and non-steroidal anti-inflammatory drugs (NSAIDs) that target the COX enzymes reduce the risk of ovarian epithelial cancers, by far the most predominant form (14), and cause growth inhibition and apoptosis in ovarian cancer cell lines (15). However, the link between morphological inhibition and reproductive inhibition is unclear.

In **Aim 1**, we used a genetic approach to determine if COX-1 deletion alters tumor formation and the rate of follicle degeneration. Cox-1 deficiency was introduced into the Wv mouse colony by crossing Wv/+ mice with Cox-1 (+/-) mice, and a new inbred colony was established by crossing Wv/+,Cox-1(+/-) siblings. Progenies homozygous for Wv and all genotypes of Cox-1, (+/+), (+/-), (-/-), were examined for ovarian morphology at 4 months. In wildtype mice, the ovaries contained follicles of all stages of development, as well as numerous corpora lutea, which develop from Graafian follicles after ovulation and indicate the ovulatory capacity of the wildtype ovaries **(Fig. 1)**.

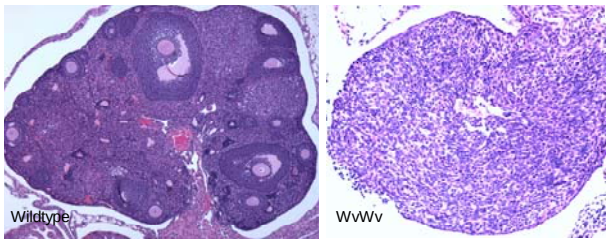

Fig. 1. Age-matched wildtype (left panel) and Wv/Wv (right panel) ovaries. Follicles of all stages and sizes are found in four-month old wildtype mouse ovaries. The Wv/Wv ovary lacks detectable follicles.

The Wv mouse ovaries were smaller, devoid of detectable follicles at any stage, and infiltrated throughout with cytokeratin- positive lesions known as tubular adenomas. Genetic deletion of COX-1 was dosage dependent, with the homozygous mutant having the most pronounced effect on the ovarian morphology, with few or negligible lesions **(Fig. 2)**. The Cox-1 deletion in the Wv/Wv background reduced the area infiltrated by the lesions nearly 3-fold. A double mutant, Wv/Wv:Cox-1(+/-):Cox-2(+/-), was as effective as the Cox-1 homozygous in reducing the tumor phenotype.

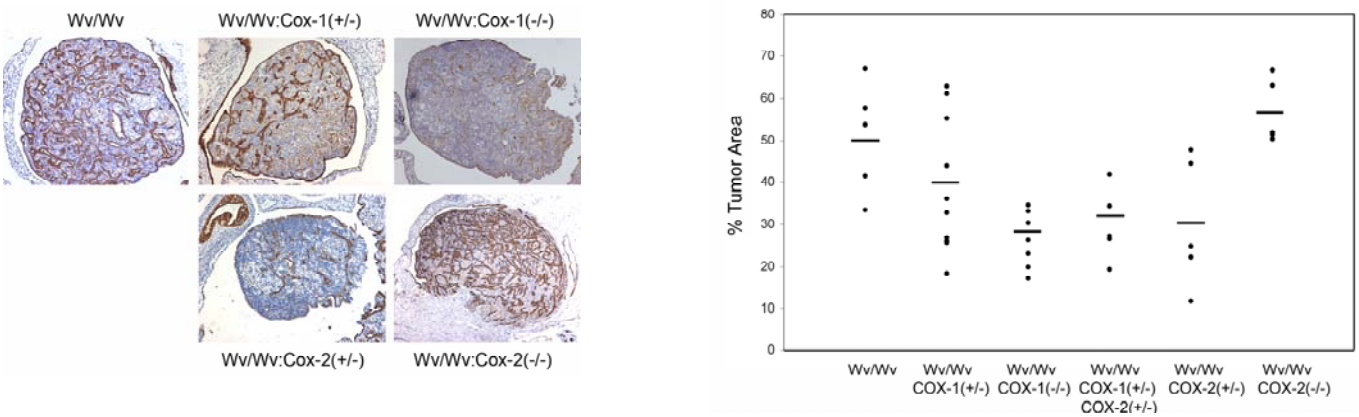

Fig. 2. (Left panel) Troma-1 (Cytokeratin-8) staining of 4-month old ovaries from mice homozygous for Wv and all genotypes of Cox-1 (+/+, +/-, -/-) shows the infiltration of epithelial tubular adenomas within the ovaries. The most significant reduction in tumor infiltration was found in the Wv/Wv:Cox-1(-/-) ovaries. **(Right panel)** The percent tumor area was determined for individual ovaries of the defined genotypes and plotted. Ovaries from Wv/Wv mice with the heterozygous Cox-1,Cox-2 genotype [Cox-1(+/-),Cox-2(+/-)] and Wv/Wv:Cox-2(+/-) genotype also show a rescue of the tumor phenotype.

We next examined the ovaries of mice at 1.5 and 3 months for follicle and tumor phenotype. The Wv/Wv:Cox-1(-/-) mice at 1.5 months contained greater primary follicles than the Wv/Wv control (**Fig. 3**) and retained follicle-like structures and lacked tumor lesions at 3 months, when the Wv/Wv ovaries were fully infiltrated with tumor, as indicated by cytokeratin-8 staining (**Fig. 4**). Thus genetic deletion of Cox-1 in the Wv/Wv background appears to slow the rate of maturation or loss of follicles, which suppresses the development of tumor lesions in the ovary. This finding is particularly interesting, since a role for cyclooxygenase-1-like activity in germ cell maturation has been reported in Drosophila ovaries (16).

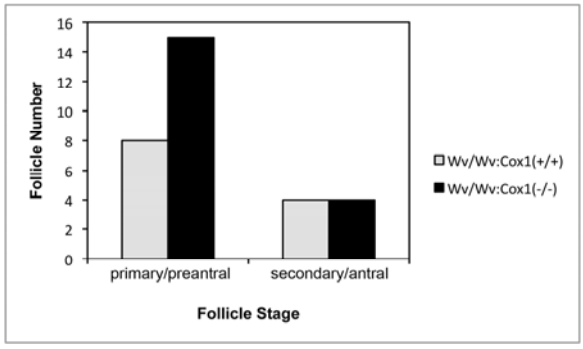

Fig. 3. Follicles were counted in images of ovarian sections taken at the largest cross-sectional diameter of the ovaries. Follicles were scored as primary/preantral or secondary/antral. The results are averages of ≥5 separate ovaries.

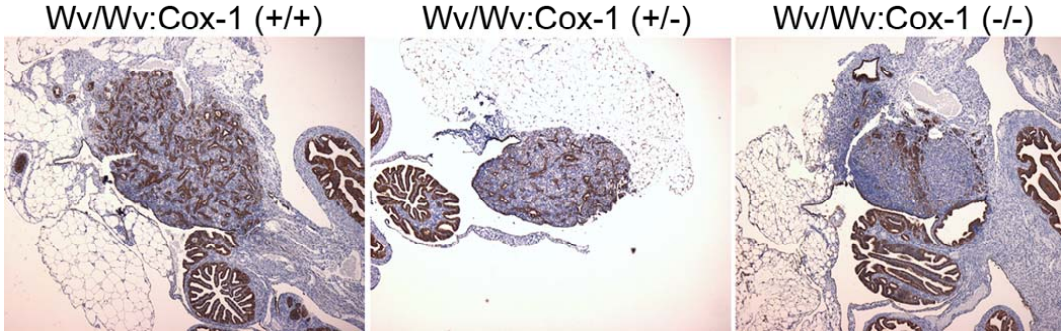

Fig. 4. Genetic deletion of Cox-1 in Wv/Wv mice delays the development of tumor lesions in the ovaries and preserves follicular structures. The effect of Cox-1 deletion was examined in the ovaries of Wv/Wv mice of 3-months of age. Cytokeratin-8 stained the epithelial components of the ovaries. Tubular adenomas fill the Wv/Wv:Cox-1(+/+) mice ovaries, but are mostly absent from Wv/Wv:Cox-1(-/-) ovaries, which also contain observable large follicular structures. The Wv/Wv:Cox-1 heterozygous mice lack the follicular structures but are marginally infiltrated with epithelial tumors.

In **Aim 2**, pharmacological cyclooxygenase inhibitors were administered to Wv/Wv mice to analyze whether Cox-1 is selective for the tumor phenotype and follicle atresia. Female Wv/Wv mice were treated with inhibitors of COX isoforms to determine that Cox-1 is the principal COX involved in ovarian morphological changes. The drugs were administered in food or drinking water for 2 months, and ovaries were collected from mice at 4 months of age. We had found just prior to the initiation of this award that Celebrex, a Cox-2 specific inhibitor, could suppress the Wv/Wv ovarian tumor phenotype (11). Here, we found that indomethacin, a non-selective COX inhibitor, and SC-560, a Cox-1 specific inhibitor, were much more effective. In animals treated with either of these two drugs, the tumor phenotype was reduced approximately 80% (from 80% to 17% tumor area). Moreover, in 11 out of 13 mice treated with Cox-1 inhibitors, one or more follicles were retained in the ovary **(Fig. 5)**.

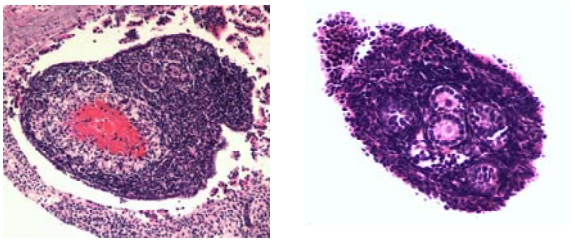

Fig. 5. Ovaries from 4-month old Wv/Wv mice treated with Cox-1 inhibitors for 2 months show a rescue of the tumor phenotype and the presence of pre-antral follicles, which are not found in non-treated Wv/Wv mice of the same age.

Based on the presence of a single layer of granulosa cells surrounding the oocyte and the size of the oocyte, the follicles appeared to be stage III-IV primary follicles (3), which is very similar to the follicles observed in the Wv/Wv:Cox-1(-/-) ovaries. Thus pharmacological inhibition of Cox-1 replicates the effect of depleting Cox-1 genetically.

Finally, in **Aim 3**, we proposed to evaluate the effect of Cox-1 depletion or inhibition on the ovarian phenotype. Our preliminary measurements showed that deletion of Cox-1 lowered serum FSH levels, though the results were not significant **(Fig. 6)**. We suspect that this slight decline reflects that by this time the Wv/Wv:Cox-1(-/-) ovaries are similar and mostly, though not entirely, devoid of follicles.

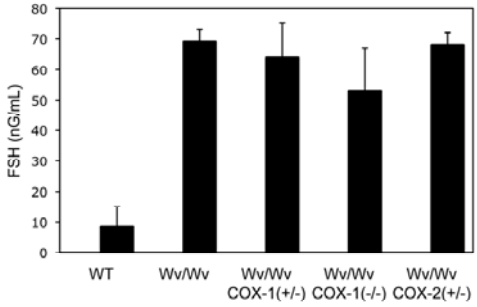

Fig. 6. Serum FSH levels in 3 month Wv/Wv mice. FSH was measured in serum obtained by retro-orbital bleed at the time of sacrifice from Wv/Wv mice of different Cox-1 genotypes, and determined by radioimmunoassay through custom service by the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA).

Key Research Accomplishments

- Found that Cox-1 genetic depletion rescues the ovarian epithelial tumor phenotype of the Wv mice.
- Determined that the effect of Cox-1 knockout is gene dosage-dependent, with the homozygous mutant having the greatest effect on ovarian morphology.
- Assessed that Cox-1 depletion delays the depletion of follicles in the younger mice and consequently the development of the epithelial lesions in the Wv mice.
- Showed that pharmacological inhibition of Cox-1 was effective in rescuing the tumor phenotype and more effective than Cox-2 inhibitors.
- Found that Cox-1 inhibition influences primary follicle preservation.

Reportable Outcomes

One manuscript was accepted during the first year of the award. A second manuscript, nearly completed, will be submitted within the next month.

Smith ER, and Xu XX. Ovarian aging, follicle depletion, and cancer: a hypothesis for the etiology of epithelial ovarian cancer involving follicle depletion. *Lancet Oncol.* 9:1108-1111, 2008.

Conclusions

These studies have examined the hypothesis that gonadotropins stimulate an inflammatory-like reaction, mediated through cyclooxygenase-1 action, which causes remodeling and morphological changes and promotes ovarian tumor development. We have utilized a practical model for post-menopausal ovarian physiology, the Wv mouse, in which cyclooxygenase-1 is over-expressed, as it is frequently found in ovarian epithelial cancers. Genetic and pharmacological inhibition of Cox-1 prevented the tumor phenotype and delayed the depletion of follicles. Importantly, the depletion of follicles may underlie the epidemiological observations linking reproductive history and ovarian cancer risk, and suggests that inhibitors of Cox-1 or NSAIDs may be more effective if administered pre-menopausally or before complete loss of follicles. The use of NSAIDs may provide a rational strategy for chemoprevention of ovarian cancer.

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1. Smith ER, and Xu XX. Ovarian aging, follicle depletion, and cancer: a hypothesis for the etiology of epithelial ovarian cancer involving follicle depletion. *Lancet Oncol.* 9:1108-1111, 2008.

List of Personnel

Elizabeth R. Smith, Ph.D. – Principal Investigator
Martin G. Belinsky, Ph.D. – Principal Investigator

Appendices

1. Smith ER, and Xu XX. Ovarian aging, follicle depletion, and cancer: a hypothesis for the etiology of epithelial ovarian cancer involving follicle depletion. *Lancet Oncol.* 9:1108-1111, 2008.

Personal View

Ovarian ageing, follicle depletion, and cancer: a hypothesis for the aetiology of epithelial ovarian cancer involving follicle depletion

Elizabeth R Smith, Xiang-Xi Xu

Lancet Oncol 2008; 9: 1108–11

Department of Medicine (E R Smith PhD, Prof X-X Xu PhD), **Sylvester Comprehensive Cancer Center** (E R Smith , Prof X-X Xu), **and Department of Obstetrics and Gynecology** (Prof X-X Xu), **University of Miami School of Medicine, Miami, FL, USA**

Correspondence to:
Dr Elizabeth R Smith, Sylvester Comprehensive Cancer Center, University of Miami School of Medicine, Miami, FL 33136, USA
esmith@med.miami.edu

The association between ovarian cancer risk and reproductive factors has been well established, and two main theories, incessant ovulation and gonadotropin stimulation, have been proposed to explain the mechanism. Recent studies using animal models of ovarian tumorigenesis, and analysis of ovarian tissues from prophylactic oophorectomies, suggest that depletion of ovarian follicles might underlie the epidemiological findings linking reproductive history and ovarian cancer risk.

Introduction

The aetiology of ovarian cancer is complex and incompletely understood, although epidemiological data decisively link ovulation frequency and reproductive hormones to ovarian cancer risk.[1–3] Increased parity and oral contraceptive use are the clearest examples of factors that decrease ovarian cancer risk, both of which limit ovulation. Two main theories, incessant ovulation[4] and gonadotropin stimulation,[5,6] have been proposed to explain the aetiology of ovulation in ovarian cancer risk,[7,8] but neither completely nor satisfactorily explains the dramatic increase in ovarian cancer incidence that occurs in the immediate postmenopausal period and that continues to rise as the ovary ages after menopause. Most (90%) ovarian cancers are derived from the surface epithelium, and nearly 85–90% of ovarian cancer develops postmenopause. Therefore, understanding how the depletion of germ cells, loss of follicular structure that surrounds the oocyte or germ cell, and cessation of ovulation, all of which define menopause, influence the development of ovarian carcinomas is of particular importance. On the basis of studies with the germ-cell-deficient Wv mice (figure 1) and examination of human ovarian tissues from prophylactic oophorectomies, we suggest that depletion of germ cells and the loss of ovarian follicular function that follows might underlie the link between reproductive factors and ovarian cancer risk.

Aetiology of ovulation in ovarian cancer

Nearly 40 years ago, Fathalla proposed the theory of incessant ovulation to clarify the association between ovulation frequency and the risk of developing epithelial ovarian cancer.[4] This hypothesis attributes the occurrence of ovarian cancer in modern day women (and domestic egg-laying hens), which is rare in other mammals, to ovulation that recurs monthly throughout the reproductive lifetime of women if not punctuated by anovulatory periods during pregnancy and breast-feeding.[4] The repetitive wounding during the release of the ovum and the cell proliferation that occurs postovulation to repair the ovarian surface epithelium have been proposed to result in mutations accumulating in the epithelial cells and ultimately the formation of tumours.[9] This central mechanism is supported experimentally in cell culture and is generally well accepted. Moreover, combined oestrogen–progesterone formulations of oral contraceptives that act by suppressing ovulation decrease the risk of ovarian cancer by about 40% after 3 years of use.[10,11] Why pregnancy and oral contraceptives provide a long-

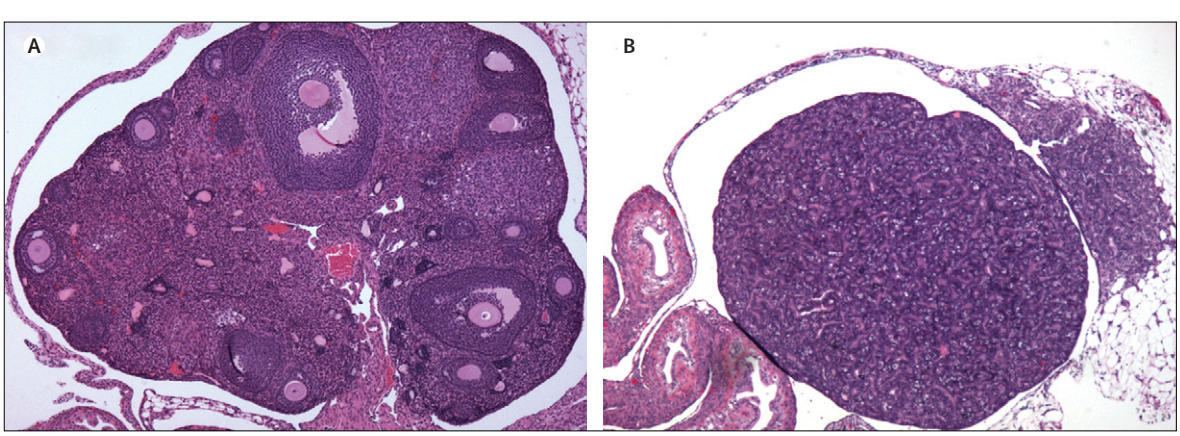

Figure 1: **Ovarian morphological changes in germ-cell-deficient Wv mice**
(A) Mature wildtype-mouse ovary contains many germ cells and follicles at various stages of development. (B) Wv ovary from a same-age mouse is depleted of germ cells and follicles and epithelial morphological changes, including tubular adenomas, permeate the ovary.

term protection, however, might be more complex than by simply limiting ovulation, because more recent studies suggest that progesterone, which is increased during pregnancy and by oral contraceptives, might also affect the clearing of transformed cells from the ovarian surface-epithelial layers.[12,13] Because the likelihood of cells carrying potentially transforming mutations increases with age, the age of either the last full-term pregnancy or the last regular use of progestin-containing oral contraceptives is a protective factor, and the benefit decreases after time.[13]

Gonadotropin stimulation in ovarian cancer risk

On the basis of the same epidemiological data, the gonadotropin stimulation theory, by contrast, postulates that surges of pituitary gonadotropins that initiate each ovulation and persist in high levels for many years after menopause also stimulate the ovarian surface-epithelial cells and induce cell transformation.[5,6] During ovulation, gonadotropins stimulate an inflammatory-like process mediated by many cytokines and proteolytic enzymes, which leads to rupture of the ovarian surface-epithelial layer for the release of the ovum.[14–16] After rupture of the follicle and the release of the ovum at the ovarian surface, the oestrogen-producing follicle is converted to a progesterone-producing corpus luteum, which then feeds back to inhibit gonadotropin levels. In menopause, which is caused by a total depletion in the number of germ cells present in the ovary and accompanied by the loss of the follicular structure that surrounds the germ cells, the feedback endocrine loop from the corpus luteum is absent, and serum gonadotropins and levels of proinflammatory cytokines are even higher in perimenopausal ovaries.[17,18] Thus, increased gonadotropin levels in postmenopausal women might foster an inflammatory environment that cannot lead to ovulation, but might contribute to ovarian cancer risk,[19] by causing either remodelling or morphological changes in the surface epithelium, which allow the transformation of genetically compromised cells and the development of cancerous lesions.[20,21] This theory seems more fitting to explain the dramatic increase in ovarian cancer incidence in the perimenopausal and postmenopausal years.[22–24] The average age of menopause, although it varies somewhat between women and cultures, is 51 years, which closely precedes the average age of ovarian cancer diagnosis, which is 54 years.

Ovarian ageing

The incidence of ovarian cancer continues to increase after menopause, and age—even more so than a family history of ovarian cancer—is the best prediction of ovarian cancer risk.[1–3] Ovaries from older women have been noted to show more morphological changes than those from younger women.[25] Presumably ovulation and subsequent repair cause these age-dependent changes, or so-called ovarian ageing,[23,26] which might represent

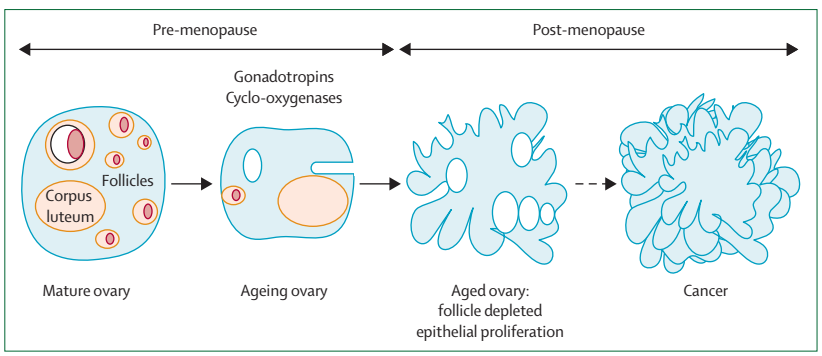

Figure 2: **Model depicting the hypothesis that germ-cell and follicle depletion underlies the aetiology of ovarian cancer risk associated with reproductive factors and menopause**

preneoplastic areas or lesions. Recently, prophylactic surgeries have allowed a closer and crucial examination of these morphological changes and potential preneoplastic lesions in healthy women.[27–29] These morphological features include papillomatosis, deep-surface invaginations, inclusion cysts, and epithelial stratifications, which are noted most frequently in perimenopausal and immediate postmenopausal ovaries of both *BRCA1/2* carriers and non-carriers,[25] and might very well be the result of ovarian ageing.

Germ-cell-deficient Wv mouse model

Recent laboratory studies of the germ-cell-deficient Wv mouse also provide intriguing ideas about the aetiology of ovarian cancer.[30] The Wv mice harbour a point mutation in the c-Kit gene that greatly decreases the tyrosine-kinase activity of c-Kit, affecting the development of germ cells, pigment cells, and mast cells.[31] Homozygous Wv-mutant mice have a similar lifespan as wildtype mice, but are essentially sterile due to the germ-cell defect.[32] Wv/Wv females contain less than 1% of the normal number of oocytes at birth, and once reproductive age is reached, ovarian follicles are rapidly depleted.[33] Subsequently, serum gonadotropins are increased and substantial epithelial morphological changes develop, including surface invaginations, inclusion cysts, papillomatosis, and benign ovarian tumours, known as tubular adenomas.[30,34] These tumours are derived from ovarian surface-epithelial cells, resembling human ovarian changes that might result from ageing.[30] In the absence of germ cells, as in the Wv mouse, suppression of gonadotropin release prevents the development of the ovarian tubular adenomas,[35] yet gonadotropin administration alone (ie, germ cells are present in the ovaries) does not result in epithelial tumours.[36,37] Moreover, transgenic mouse models targeting the gonadotropin pathway without depletion of oocytes do not develop epithelial tumours. Female mice overexpressing follicle-stimulating hormone (FSH) in levels far exceeding those in postmenopausal women develop haemorrhagic and cystic ovaries, but do not develop epithelial lesions.[38] Additionally, genetic knockouts of the FSH receptor in

the ovary, in which circulating serum FSH levels are high but non-functioning, do not develop epithelial cancers, but instead sex-cord tumours, which represent only a small subset of human ovarian cancers.[39] Thus, the early depletion of germ cells and subsequent increase in gonadotropins in the Wv mice might constitute a relevant, albeit exaggerated model, mimicking the postmenopausal biology and ovarian morphological ageing in women.

Follicle depletion

Studies of the Wv mice prompted us to postulate that depletion of ovarian germ cells and follicles might underlie the aetiology of ovarian cancer risk associated with reproductive factors and menopause status (figure 2), and might in fact unify incessant ovulation and gonadotropin stimulation as mechanisms.

Follicle depletion explains the age-dependent risk of ovarian cancer: ovarian cancer generally develops in the immediate postmenopausal years, when ovarian follicles are depleted. The depletion of ovarian follicles obviously precedes and causes increased serum gonadotropins, which stimulate an inflammatory environment in the ovary that is permissive to transformation of surface epithelial cells and tumour development. Furthermore, both incessant ovulation and gonadotropin stimulation will accelerate the depletion of ovarian follicles. Additionally, protective factors, such as the use of birth-control pills,[40] and cyclo-oxygenase inhibitors,[41–43] might preserve follicles. Cyclo-oxygenases participate in follicle development and ovulation,[44,45] and their inhibition might slow follicle maturation and extend follicle lifespan. Findings show that lifetime ovulation correlates with premenopausal, but not postmenopausal, ovarian cancer risk,[46] and a decrease of pituitary gonadotropin release with hormone-replacement therapy does not decrease ovarian cancer risk.[47] These findings also support the notion that the presence of ovarian follicles, rather than ovulation and gonadotropin stimulation, is a major determinant of ovarian cancer risk.

Conclusion

The follicle-depletion hypothesis predicts that follicle preservation and delaying reproductive ageing might prevent ovarian cancer or decrease the risk of developing this disease, and that menopause timing might correlate with ovarian cancer incidence, which can be verified by epidemiological studies designed to assess these factors.

Search strategy and selection criteria

Information for this Personal View was obtained by searches of PubMed using the search terms: "ovarian cancer", "menopause", "follicle depletion", "gonadotropins", "ovarian cancer risk", and "Wv mice". Only papers published in English between 1957 and 2007 were included.

Conflicts of interest

The authors declared no conflicts of interest.

Acknowledgments

We are grateful to colleagues in the Ovarian Cancer Program at Fox Chase Cancer Center (Philadelphia, PA, USA) for their suggestions for the development of our research and ideas. We especially thank laboratory members, Kathy Qi Cai, Wan-Lin Yang, and Jennifer Smedberg, for their work, on which the ideas in this paper are based. These studies were supported by grants R01 CA099471 from the US National Cancer Institute and the US National Institutes of Health, and W81XWH-06-1-0095 from the US Department of Defense (X-XX), and grants from the Marsha Rivkin Ovarian Cancer Research Foundation (Seattle, WA, USA), and the Department of Defense Ovarian Concept Award W81XWH-07-1-0303 (ERS). Additional funding from the Teal Ribbon Ovarian Cancer Research Foundation (Philadelphia, PA, USA) is greatly appreciated.

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